# DESCRIPTION

## MATERIALS AND METHODS FOR IMPROVED SWEET CORN

This invention was made with government support under USDA grant number 9801006. The government has certain rights in the invention.

### Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application Serial No. 60/516,088, filed October 31, 2003, the disclosure of which is incorporated herein by reference in its entirety.

### Background of the Invention

ADP glucose pyrophosphorylase (AGP) is an important starch biosynthesis enzyme in plants. AGP catalyzes the conversion of ATP and α-glucose-1-phosphate to ADP-glucose and pyrophosphate. ADP-glucose is used as a glycosyl donor in starch biosynthesis by plants and in glycogen biosynthesis by bacteria. The importance of ADP-glucose pyrophosphorylase as a key enzyme in the regulation of starch biosynthesis was noted in the study of starch deficient mutants of maize (*Zea mays*) endosperm (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). Biochemical and genetic evidence has identified AGP as a key enzyme in starch biosynthesis in higher plants and glycogen biosynthesis in *E. coli* (Preiss *et al.*, 1994; Preiss *et al.*, 1996). AGP catalyzes what is viewed as the initial step in the starch biosynthetic pathway with the product of the reaction being the activated glucosyl donor, ADPglucose. This is utilized by starch synthase for extension of the polysaccharide polymer (reviewed in Hannah 1996).

Initial studies with potato AGP showed that expression in *E. coli* yielded an enzyme with allosteric and kinetic properties very similar to the native tuber enzyme (Iglesias *et al.*, 1993; Ballicora *et al.*, 1995). Greene *et al.* (1996a, 1996b) showed the usefulness of the bacterial expression system in their structure-function studies with the potato AGP. Multiple mutations important in mapping allosteric and substrate binding sites have been identified (Okita *et al.*, 1996).

AGP enzymes have been isolated from both bacteria and plants. Bacterial AGP consists of a homotetramer, whereas plant AGP from photosynthetic and non-photosynthetic

tissues is a heterotetramer composed of two different subunits. The plant enzyme is encoded by two different genes, with one subunit being larger than the other. This feature has been noted in a number of plants. The AGP subunits in spinach leaf have molecular weights of 54 kDa and 51 kDa, as estimated by SDS-PAGE. Both subunits are immunoreactive with antibody raised against purified AGP from spinach leaves (Copeland *et al.*, 1981; Morell *et al.*, 1988). Immunological analysis using antiserum prepared against the small and large subunits of spinach leaf showed that potato tuber AGP is also encoded by two genes (Okita *et al.*, 1990). The cDNA clones of the two subunits of potato tuber (50 and 51 kDa) have also been isolated and sequenced (Muller-Rober *et al.*, 1990; Nakata *et al.*, 1991). The large subunit of potato tuber AGP is heat stable (Nakata *et al.*, 1991, *supra*).

As Hannah and Nelson (1975, 1976) postulated, both *Shrunken-2* (*Sh2*) (Bhave *et al.*, 1990) and *Brittle-2* (*Bt2*) (Bae *et al.*, 1990) are structural genes of maize endosperm ADP-glucose pyrophosphorylase. *Sh2* and *Bt2* encode the large subunit and small subunit of the enzyme, respectively. Based on cDNA sequencing, *Sh2* and *Bt2* proteins have predicted molecular weight of 57,179 Da (Shaw *et al.*, 1992) and 52,224 Da, respectively. The endosperm is the site of most starch deposition during kernel development in maize. *Sh2* and *Bt2* maize endosperm mutants have greatly reduced starch levels corresponding to deficient levels of AGP activity. Mutations of either gene have been shown to reduce AGP activity by about 95% (Tsai and Nelson, 1966, *supra*; Dickinson and Preiss, 1969, *supra*). Furthermore, it has been observed that enzymatic activities increase with the dosage of functional wild type *Sh2* and *Bt2* alleles, whereas mutant enzymes have altered kinetic properties. AGP is the rate limiting step in starch biosynthesis in plants. Stark *et al.* (1992) placed a mutant form of *E. coli* AGP in potato tuber and obtained a 35% increase in starch content.

The cloning and characterization of the genes encoding the AGP enzyme subunits have been reported for various plants. These include *Sh2* cDNA (Bhave *et al.*, 1990, *supra*), *Sh2* genomic DNA (Shaw *et al.*, 1992, *supra*), and *Bt2* cDNA (Bae *et al.*, 1990, *supra*) from maize; small subunit cDNA (Anderson *et al.*, 1989) and genomic DNA (Anderson *et al.*, 1991) from rice; and small and large subunit cDNAs from spinach leaf (Morell *et al.*, 1988, *supra*) and potato tuber (Muller-Rober *et al.*, 1990, *supra*; Nakata *et al.*, 1991, *supra*). In addition, cDNA clones have been isolated from wheat endosperm and leaf tissue (Olive *et al.*, 1989) and *Arabidopsis thaliana* leaf (Lin *et al.*, 1988). AGP sequences from barley have also been described in Ainsworth *et al.* (1995).

AGP has been found to function as an allosteric enzyme in all tissues and organisms investigated to date. The allosteric properties of AGP were first shown to be important in *E. coli*. A glycogen-overproducing *E. coli* mutant was isolated and the mutation mapped to the structural gene for AGP, designated as *glg*C. The mutant *E. coli*, known as *glg*C-16, was shown to be more sensitive to the activator, fructose 1,6 bisphosphate, and less sensitive to the inhibitor, cAMP (Preiss 1984). Although plant AGP's are also allosteric, they respond to different effector molecules than bacterial AGP's. In plants, 3-phosphoglyceric acid (3-PGA) functions as an activator while phosphate ($PO_4$) serves as an inhibitor (Dickinson and Preiss, 1969, *supra*).

There is a great demand for sweeter sweet corn. This is particularly true in Florida where the time from harvest to consumption is several to many days. Traditional sweet corns utilized a mutant recessive allele of the gene termed *sugary1 (su1)*. U.S. Patent No. 6,410,716 describes isolation of maize *sugary1* gene. In the 1960s, John Laughnan at the University of Illinois pointed out the advantages of a recessive mutant allele of *shrunken2 (sh2)*. The mutant *sh2* allele conditions two- to four-fold more sugar than does the mutant *su1*. Corns with the *sh2* allele maintain their sweetness (as detected by people) much longer. One consequence of this was the release from Florida of corns like 'Florida Stay Sweet'.

One problem with traditional sweet corn breeding programs has been that the mutations that result in high sugar and low starch levels are often recessive (thus, plants producing ears destined for consumption must be homozygous for the recessive allele) and, therefore, must be present in both of the inbred lines used to create commercial seed. Hence, breeders have to deal with poor seed germination and seedling vigor in two lines of inbreds. For example, the downside to *sh2* "super-sweet corns," as they are called in the trade, is reduced germination and reduced seedling vigor. Much effort has gone into improvement of these traits by traditional breeding, as well as through the use of a leaky *sh2* allele, designated *sh2-i* (U.S. Patent No. 6,184,438; Giroux and Hannah (1994)), and the use of late promoters to turn the wild type gene on late in kernel development. The mutant *sh2-i* is so named because it gives an intermediate or leaky phenotype, in which limited AGPase activity is present. Accordingly, starch levels are lower and sucrose levels are higher in the mutant than in a wild-type line. Giroux and Hannah (1994) showed that this particular mutant was caused by a point mutation in the AG dinucleotide at the end of intron 2. This causes skipping of exon 3 during pre-mRNA processing. However, because exon 3 is 123 base pairs in length (a multiple of 3) the reading frame of the mRNA is conserved. It was originally thought that

AGPase resulting from the skipping of exon 3 maintained some activity, resulting in the intermediate phenotype. However, it was shown that sites contained in exon 3 are crucial for AGPase function in potato tubers (Greene *et al.*, 1996a). On further analysis, it was found that the skipping of exon 3 in the *sh2-i* allele only occurs in 90% of the transcripts. In the remaining 10% of transcripts, normal splicing occurs, giving rise to functional AGPase, though in far less concentration compared to wild-type *Sh2* alleles (Lal *et al.*, 1999).

## Brief Summary of the Invention

The subject invention pertains to materials and methods for producing improved sweet corn. Sweet corn produced according to the present invention exhibits increased sweetness without a reduction in germination or seedling vigor typically associated with the production of most sweet corns. One aspect of the invention pertains to a dominant loss-of-function (silenced) *sh2* allele and plants, plant tissue, and plant seeds having at least one copy of the allele. In an exemplified embodiment, maize having a dominant loss-of-function *sh2* allele of the invention was obtained following transformation of maize cells with constructs driven by a *Sh2* promoter and containing a polynucleotide encoding a large subunit of AGP that comprises the *Rev6* mutation and the heat stable *HS33* mutation, and having the *Sh1* first intron, and also containing a *Nos* terminator. Transformants exhibiting the desired characteristics were then identified and further evaluated.

Another aspect of the invention pertains to a mixture of corn seed that provides for corn plants that produce improved sweet corn via increased sucrose content of corn kernels. A further aspect of the invention concerns methods for producing corn plants with increased sucrose content.

## Brief Description of the Drawings

**Figure 1** shows a comparison of sucrose levels in wild type selfed vs. wild type crossed to *Sh2* Silencer. The sharp contrast between a line with a functional *Sh2* and a silenced *Sh2* is apparent here. Comparative sucrose levels shown for each day post pollination are the average of three ears, except where denoted in Table 2. Numerical data are found in Table 3.

**Figure 2** shows a comparison of sucrose levels in *shrunken2-i* selfed vs. *shrunken2-i* crossed to *Sh2* Silencer. Silencing effects on an intermediate phenotype is apparent. Data for the *sh2iX* at 25dpp is considered spurious since only one ear was harvested. Other points for

which three ears were not available are denoted in Table 2. Numerical data are found in Table 3.

**Figure 3** shows a comparison of sucrose levels in *sugary* selfed vs. *sugary* crossed to *Sh2* Silencer. Comparative sucrose levels shown for each day post pollination are the average of three ears, except where denoted in Table 2. Numerical data are found in Table 3.

**Figure 4** shows a comparison of sucrose levels in *shrunken2* selfed vs. *shrunken2* crossed to *Sh2* Silencer. Comparative sucrose levels shown for each day post pollination are the average of three ears, except where denoted in Table 2. Numerical data are found in Table 3.

## Detailed Disclosure of the Invention

The subject invention pertains to materials and methods for producing improved sweet corn. Sweet corn produced according to the present invention exhibits increased sweetness without a reduction in germination or seedling vigor. One aspect of the invention pertains to a dominant loss-of-function (silenced) *sh2* allele and plants, plant tissue, and plant seeds having at least one copy of the allele. Another aspect of the invention pertains to a mixture of corn seed that provides for corn plants that produce improved sweet corn. A further aspect of the invention concerns methods for producing improved sweet corn plants having increased sucrose content in their kernels relative to wild type corn or corn that does not have a dominant loss-of function *sh2* allele by growing a corn seed mixture of the present invention or by selective planting of corn seed of the invention followed by detasseling of specific corn plants.

In one embodiment of the subject invention, a mixture of corn seed is provided wherein corn seed from a first corn line having a dominant loss-of-function *sh2* allele of the invention is mixed with corn seed from a second corn line that is genetically male sterile and having a functional or semi-functional *sh2* allele or a wild type *Sh2* allele. Optionally, the second corn line can contain genes that provide for good eating attributes, such as, for example, a thin pericarp (Abedon *et al.*, 1999). In addition, both the first and second corn lines can optionally be homozygous for a recessive *sugary* (*su1*) allele and/or contain the *sh2-i* allele. Corn lines that comprise the *su1* allele include the commercially available "Silver Queen," "Argent," and "Quickie." Corn lines that comprise the *sh2-i* allele are commercially available from Syngenta Seeds (Golden Valley, MN). The seed from the first corn line can comprise from about 0.1% to about 50% of the corn seed mixture; thus, the seed from the

first corn line can comprise about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, or 50% of the corn seed mixture. In one embodiment, the seed from the first corn line can comprise from about 1% to about 50% of the corn seed mixture, or from about 1% to about 25% of the corn seed mixture, or from about 1% to about 15% of the corn seed mixture. In one embodiment, the seed from the first corn line comprises from about 1% to about 10% of the corn seed mixture. In a specific embodiment, the seed from the first corn line makes up about 5% of the seed mix and seed from the second corn line makes up about 95% of the seed mix. The percentage of seed in the mixture can be based on seed weight or seed number.

Because of male sterility, all pollinations would be with pollen from the line having the dominant loss-of-function *sh2* allele. Also, because of the dominant nature of the *sh2* allele from the first line, all resulting kernels in the pollinated plants will contain high sugar levels. The second line will germinate well if it contains at least one copy of the functional or semi-functional *Sh2* allele. The seed that contains the dominant loss-of-function *sh2* allele would probably show reduced germination. However, this seed makes up only a small percentage of the total seed mixture. For example, if the seed mix contained 5% first line seed and 95% second line seed, and only 50% of the first line germinated and 98% of the second line germinated, the grower would still garner greater than 95% total germination.

The subject invention also concerns methods of producing corn with increased sucrose content. In one embodiment, a corn seed mix of the present invention is planted and corn plants grown therefrom. The seed of the first corn line contains a dominant loss-of-function *sh2* allele. The seed of the second corn line contains a functional or semi-functional *Sh2* allele and corn grown from the seed of the second corn line is male sterile or can be made male sterile. Because the second corn line in the methods of the subject invention is male sterile, only the first corn line can act as a pollinator of the second corn line. The second corn line used in the corn mix can be genetically male sterile or can be made male sterile by any suitable method including, but not limited to, mechanical or hand detasseling, or chemical means. In those embodiments where the second corn line is made male sterile by detasseling, those of ordinary skill in the art can readily determine when during growth of the corn plant the tassels should be removed to eliminate pollen shedding. In one embodiment, the second corn line is genetically male sterile. In those embodiments where the second corn

line is made male sterile by non-genetic means, then the first and second corn lines must be planted in separate rows so that the two lines can be readily distinguished from each other.

In those embodiments of the subject methods wherein the second corn line is genetically male sterile, the corn seed mix used in the subject method can comprise from about 0.1% to about 50% of the corn seed mixture; thus, the seed from the first corn line can comprise about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, or 50% of the corn seed mixture. In one embodiment, seed of the first corn line comprises from about 1% to about 50% of the first corn line, or from about 1% to about 25% of the corn seed mixture, or from about 1% to about 15% of the corn seed mixture. In one embodiment, the seed of the first corn line comprises from about 1% to about 10% of the corn seed mixture and the seed of the second corn line comprises from about 99% to 90% of the corn seed mix. In a specific embodiment, the seed from the first corn line makes up about 5% of the seed mix and seed from the second corn line makes up about 95% of the seed mix. Following pollination and maturation of the corn plants grown from the methods of the invention, the corn ears are harvested at a suitable time.

In another embodiment, corn with increased sucrose content can be produced by planting one row of a first corn seed that has a dominant loss-of-function *sh2* allele of the present invention, followed by planting one or more rows of a second corn seed that has a functional or semi-functional *sh2* or wild type *Sh2* allele, then a row of the first corn seed, and then one or more rows of the second corn seed, and so on. After planting, corn is allowed to grow. The tassel on the corn plants grown from the second corn seed is removed prior to pollen shedding. Thus, only pollen from the corn plants having the dominant loss-of-function *sh2* allele can pollinate the detasseled corn plants grown from the second corn seed. In one embodiment, 6 to 8 rows of the second corn seed is planted, followed by a single row of the first corn seed, followed by 6 to 8 rows of the second corn seed, and so on in an alternating manner. Optionally, the second corn seed can contain one or more genes that provide for desirable eating attributes or phenotype, such as a thin pericarp. Both the first and second corn seed can also optionally be homozygous for a recessive *surgary* (*su1*) allele and/or contain the *sh2-i* allele.

The subject invention also concerns corn plants, plant tissue, and seed comprising a dominant loss-of-function *sh2* allele. Corn plants having a dominant loss-of-function *sh2* allele can be prepared using standard gene silencing methods, including transcriptional silencing and post-transcriptional gene silencing, to prepare transformed and transgenic plants wherein expression of an *Sh2* or functional or semi-functional *sh2* gene is silenced. Methods for silencing expression of a targeted gene include, but are not limited to, methods described in U.S. Patent Nos. 6,573,099 and 6,506,559; in published International Publication Nos. WO 01/01751 and WO 02/044321; and in U.S. Publication Nos. US 2003/0108923 and US 2002/0086356. Constructs comprising a polynucleotide encoding a large subunit of AGP, or a fragment thereof, can be used to transform plant cells. Plants grown from the transformed cells are screened for silencing of *shrunken2* expression. In one embodiment, plants with a dominant, loss-of-function *sh2* allele were prepared by transforming maize plant cells with sense constructs comprising maize endosperm *Sh2* gene containing both a *Rev6* mutation (U.S. Patent Nos. 5,589,618, 5,650,557, and 5,872,216) and an *HS 33* mutation (U.S. Patent Nos. 6,403,863 and 6,069,300), and also comprising the *Sh2* promoter, the *Sh1* first intron, and the *Nos* terminator using the protocols described in U.S. application Serial No. 60/516,088, and described at the "Protocols" hyperlink which can be found at the Worldwide Website: www.agron.iastate.edu/ptf/Web/mainframe.htm#, the disclosure of which is incorporated herein by reference in its entirety. The *HS33* mutation conditions a change from a histidine to a tyrosine at position 333 of maize endosperm AGP large subunit amino acid sequence.

Corn seed (designated as "Sil.Sh2") comprising a dominant loss-of-function *sh2* allele was deposited with American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, on November 1, 2004. The subject corn seed has been deposited under conditions that assure that access to the seed will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposit will be available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject seed deposit will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, *i.e.*, it

will be stored with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the seed. The depositor acknowledges the duty to replace the deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject seed deposit will be irrevocably removed upon the granting of a patent disclosing it.

Corn plants comprising a dominant loss-of-function *sh2* allele can be prepared by growing plants from the corn seed of the present invention. The corn plants can then be bred with other corn lines to introduce the dominant loss-of-function *sh2* allele into other desirable corn plant genetic backgrounds using standard maize breeding methods. Thus, the subject invention encompasses any corn plant of any genetic background that comprises a dominant, loss-of-function *sh2* allele.

The subject invention also concerns corn plants, plant tissue and seeds produced according to any of the methods of the present invention. Optionally, the corn plants, plant tissue, and seeds of the invention are homozygous for a recessive *sugary* (*su1*) allele and/or the *sh2-i* allele. In one embodiment, a corn plant, plant tissue or plant seed comprises one or more genes that provide for desirable eating attributes or phenotype of corn ears.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1

Corn homozygous for the dominant loss-of-function *sh2* allele was independently crossed onto four types of corn:

(1) *sh2* (standard, null mutant allele that conditions very high levels of sugar);

(2) *sh2-i* (exhibits great germination, and very high sugars compared to standard sweetcorns, but not as high as the standard, null mutant allele);

(3) *sugary* (*su*, standard sweetcorn, *i.e.*, Silver Queen); and

(4) wild type (standard field corn).

Each of the four lines below was also self pollinated. Ear samples were then taken at 14 days, 18 days, and 25 days after pollination. The middle harvest, at 18 days after pollination, is about when people normally eat corn.

Sugar levels in the crossed ears were divided by sugar levels in the selfed ears and the ratios are presented in Table 1 below.

**Table 1.** Fold increase in total sugars conditioned by the silenced *Sh2*

| Date post pollination | 14 | 18 | 25 | average |
|---|---|---|---|---|
| *sh2* | 0.82 | 1.07 | 1.17 | 1.02 |
| *sh2-i* | 1.5 | 1.37 | 3.01 | 1.96 |
| *sugary* | 2.64 | 2.62 | 4.70 | 3.32 |
| wild type | 3.2 | 7.2 | 3.96 | 4.79 |

The Table shows the following: (1) Sugar levels of the silenced *Sh2* are comparable to those found in corn having the standard, null, very high sugar allele (*sh2*), (2) sugar levels in the corn from the cross with the dominant loss-of-function *sh2* allele are higher than those in *sh2-I* especially late in development and (3) the sugar levels in the corn from the cross with the dominant loss-of-function *sh2* allele run 2.5 to 7 fold above sugar levels found in wild type and the standard sweet corn, *sugary* (Silver Queen was used as the *sugary* source.)

Example 2

Sugar Extraction. Each of the eight genotypes was given a number reference: 1, WT (wild type); 2, WT crossed with the silencer; 3, *sh2i*; 4, *sh2i* crossed with the silencer; 5, *su*; 6, *su* crossed with the silencer; 7, *sh2*; 8, *sh2* crossed with the silencer. "Wild type" refers to a maize line not lacking any specific gene function and hence it normally synthesizes starch rather than building up sugar. "Silencer" refers to a maize line that contains a dominant silenced (loss-of-function) *sh2* allele. Three ears were harvested at 14, 18, and 25 days post pollination (dpp), respectively, for each genotype, with the following exceptions: genotype 8 had no ears at 25 dpp; genotype 4 had only one ear at 25 dpp; genotypes 1 and 2 had only two ears at 14 dpp; genotypes 1, 2, and 7 had only 2 ears at 18 dpp; genotypes 2 and 7 had only two ears at 25 dpp (Table 2). Individual ears of the same genotype that were harvested at the

same day post-pollination were given letter designations (A, B, or C). Kernels from each ear were removed in the field and individually stored on dry ice immediately. Long-term storage of kernel tissue was at -80° C.

For extraction, 5 g samples of kernel tissue were ground in liquid nitrogen using mortar and pestle. Samples were suspended in 21 mL of 95% ethanol (heated to 65° C) in 50 mL Oakridge tubes. Tubes were then covered with large glass marbles, and samples were placed in a 65° C water bath for 15 minutes. Samples were then centrifuged at 17,000 *g* for 5 minutes. The supernatants were saved, and pellets were resuspended in 25 mL of 80% ethanol. Again, samples were centrifuged at 17,000 *g* for 5 minutes, and the supernatants were saved. The supernatants of each sample were then combined and brought to 50 mL with 80% ethanol. For ease of storage, 20 mL of each extract was stored in a scintillation vial at 4° C.

To remove lipids, saved extracts were washed with two subsequent treatments of 10 mL HPLC grade hexane in a 250 mL separation funnel.

Sucrose Determination. Each sample was tested in three trials, using three replicates, giving a total of 27 data points for each genotype at each harvest (with the exception of aforementioned genotypes without three ears). To determine the amount of sucrose in each sample, an adaptation of the Nelson-Somogyi Assay for reducing sugars was used (Somogyi 1952), in conjunction with invertase digestion. In doing so, an undigested replicate could be assayed with a spectrophotometer to measure the reducing sugars in the sample. All sucrose in a sample digested with invertase would be converted into reducing sugar (glucose and fructose), and thereby give a higher reading in the Nelson-Somogyi assay. From the differences in readings between digested and undigested replicates, the amount of sucrose present in the original sample was calculated. For each replicate of a sample, aliquots were diluted in distilled water (*dd*$H_20$) to put concentrations in the measurable range of the assay (0-100 μg/mL).

The first aliquot of each sample was 15 μL and was diluted into 495 μL of $H_20$. The second aliquot was 5 μL and was diluted into 495 μL of $H_20$ and 10 μL of invertase solution (2.0 ng/mL dissolved in *dd*$H_20$). All samples were placed in a water bath at 37°C overnight to allow complete digestion with invertase. After overnight digestion, 500 μL of each replicate was mixed in a 10 mL glass culture tube with 1.0 mL of the Nelson-Somogyi Copper Reagent. Glass marbles were placed on the culture tubes to reduce evaporative loss, and then the replicates were placed in a boiling water bath for 15 minutes. After cooling to

room temperature, 1.0 mL of the Nelson-Somogyi Arsenomolybdate Reagent was mixed with each replicate.

Absorbance readings were then measured in a spectrophotometer at 520 nm. A standard curve was also assayed with glucose standards at 0 μg/mL (*dd*$H_2O$), 10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL for each set of replicates. Reducing sugar concentrations of replicates were extrapolated from the standard curve using linear regression software found in GraphPad Prism (GraphPad Software, Inc.).

**Table 2.** Number of ears harvested. Attempts were made to harvest 3 ears for each genotype at each day post-pollination; however, this was not always possible. Genotype abbreviations indicate the following: WT, Wild type (*Sh2*); *sh2-i*, *shrunken2-I*; *su*, *sugary*; *sh2*, *shrunken2*. Abbreviations followed by an "X" indicated being crossed by silenced *shrunken2*.

| Assigned Number | Genotype | Days Post-Pollination at Harvest | Number of Ears Harvested |
|---|---|---|---|
| 1 | WT self | 14 | 2 |
| 1 | WT self | 18 | 2 |
| 1 | WT self | 25 | 3 |
| 2 | WTX Sil.Sh2 | 14 | 2 |
| 2 | WTX Sil.Sh2 | 18 | 2 |
| 2 | WTX Sil.Sh2 | 25 | 3 |
| 3 | *sh2-i* self | 14 | 3 |
| 3 | *sh2-i* self | 18 | 3 |
| 3 | *sh2-i* self | 25 | 3 |
| 4 | *sh2-i*X Sil.Sh2 | 14 | 3 |
| 4 | *sh2-i*X Sil.Sh2 | 18 | 3 |
| 4 | *sh2-i*X Sil.Sh2 | 25 | 1 |
| 5 | *su* self | 14 | 3 |
| 5 | *su* self | 18 | 3 |
| 5 | *su* self | 25 | 3 |
| 6 | *su*X Sil.Sh2 | 14 | 3 |
| 6 | *su*X Sil.Sh2 | 18 | 3 |
| 6 | *su*X Sil.Sh2 | 25 | 3 |
| 7 | *sh2* self | 14 | 3 |
| 7 | *sh2* self | 18 | 2 |
| 7 | *sh2* self | 25 | 2 |
| 8 | *sh2*X Sil.Sh2 | 14 | 3 |
| 8 | *sh2*X Sil.Sh2 | 18 | 3 |
| 8 | *sh2*X Sil.Sh2 | 25 | 0 |

After extrapolation of reducing sugar concentrations, and subsequent calculation of sucrose concentrations, the following averages for each genotype at each day post-pollination were found (Table 3). All concentrations are milligrams of sucrose per gram of fresh kernel tissue.

**Table 3.** Average sucrose concentrations. Average sucrose concentrations for each genotype at each day post-pollination are given as milligrams sucrose per gram of fresh kernel tissue. Standard deviations are given in parentheses beneath each concentration.

| dpp | 1 (*WT*) | 2 (*WTX*) | 3 (*sh2-i*) | 4 (*sh2-iX*) | 5 (*su*) | 6 (*suX*) | 7 (*sh2*) | 8 (*sh2X*) |
|---|---|---|---|---|---|---|---|---|
| 14 | 4.45 (4.87) | 36.13 (1.49) | 31.30 (9.78) | 40.28 (4.09) | 14.79 (3.87) | 39.34 (7.04) | 46.19 (3.67) | 51.66 (7.60) |
| 18 | 13.18 (3.67) | 46.83 (10.34) | 28.64 (3.87) | 48.00 (12.45) | 9.15 (3.49) | 58.45 (3.22) | 66.23 (4.70) | 39.46 (7.60) |
| 25 | 2.39 (1.39) | 31.81 (8.31) | 23.36 (13.41) | 74.88 (No SD) | 6.18 (3.11) | 37.45 (8.10) | 35.15 (4.69) | |

Wild type. The average sucrose concentrations (with standard deviations) for the self-pollinated wild type ears at 14, 18, and 25 dpp were 4.45 (4.87), 13.18 (3.67), and 2.39 (1.39) mg/g, respectively. These are in sharp contrast to the ears resulting from the cross with the silencer, which were 36.13 (1.49), 46.83 (10.34), and 31.81 (8.31) mg/g at 14, 18, and 25 dpp, respectively (Figure 1).

*shrunken2-i*. For the self-pollinated *shrunken2-i* ears, the sucrose concentrations were 31.30 (9.78), 28.64 (3.87), and 23.36 (13.41) mg/g at 14, 18, and 25 dpp, respectively. Crossed ears had sucrose concentrations of 40.28 (4.09), 48.88 (12.45), and 74.88 (no std. dev.) mg/g at 14, 18, and 25 dpp, respectively (Figure 2).

*sugary*. The sucrose concentration of self-pollinated *sugary* ears were 14.79 (3.87), 9.15 (3.49), and 6.18 (3.11) mg/g at 14, 18, and 25 dpp, respectively. Ears from *sugary* crossed with the silencer gave sucrose concentrations at 39.34 (7.04), 58.45 (3.22), and 37.45 (8.10) mg/g at 14, 18, and 25 dpp, respectively (Figure 3).

*shrunken2*. Sucrose concentrations of self pollinated *shrunken2* ears were 46.19 (3.67), 66.23 (4.70), and 35.15 (4.69) mg/g at 14, 18, and 25 dpp, respectively. Concentrations of crossed ears were 51.66 (7.60) and 39.46 (3.50) at 14 and 18 dpp, respectively (Figure 4).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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